Nitric Oxide and Abscisic Acid Cross Talk in Guard Cells[1]

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Despite recent efforts to elucidate the regulation of stomatal movement, many components within the branched pathways of guard cell abscisic acid (ABA) signaling remain to be identified. Here, we show results supporting the involvement of nitric oxide (NO) as a new component of this signaling pathway.

NO is a short life bioactive molecule first described as a toxic compound, but now recognized to be an important signal and effector molecule both in animal and plant cell physiology. Even though NO research in plants is not as advanced as in animals, in the last decade NO was proved to participate in many key physiological processes such as growth, pathogen defense reaction, development, programmed cell death, and stress tolerance (Foissner et al., 2000; Pedroso et al., 2000; Beligni and Lamattina, 2001a). In plants, as in animals, NO was proved to interact with other signaling elements such as cADPR, lipids, cGMP, ion channels, Ca^{2+}, and others. In addition, much evidence is appearing lately about cross talk between NO and some plant hormones during adaptive responses to adverse conditions (Hausladen and Stamler, 1998; Durner and Klessig, 1999; Jacob et al., 1999; Beligni and Lamattina, 2001b; Wendehenne et al., 2001). Furthermore, it is now becoming clear that a network constituted at many levels is operating during plant responses to stress stimulus (Knight and Knight, 2001).

ABA is one of the most studied phytohormones due to its key participation in different physiological events throughout the whole plant cycle. Under drought stress conditions, ABA accumulates in leaf tissue, generating a net loss of guard cell turgor that led to stomatal closure, thus reducing transpirational water loss. ABA-induced stomatal closure involves a net increase in guard cell cytoplasmic Ca^{2+} concentrations. Furthermore, cADPR, ryanodine receptors, and phospholipases C and D have been also involved as second messengers in this signaling pathway (MacRobbie, 1998; Jacob et al., 1999; Sanders et al., 1999; Schroeder et al., 2001b). Even though NO was recently reported to induce stomatal closure in *Vicia fava* epidermal strips, there is no information about how NO induces this response (García Mata and Lamattina, 2001). In this work, we provide strong evidence on the existence of an NO-mediated action in ABA-dependent stomatal closure.

V. fava epidermal strips were treated with increasing concentrations of ABA in the presence of increasing concentrations of the NO releaser sodium nitroprusside (SNP). As expected, both ABA and SNP induced stomatal closure in a dose-dependent manner. However, at the higher concentration assayed (1 and 10 μM ABA; 10 and 100 μM SNP), the stomatal closure percentage of the combined treatment was significantly lower than in those experiments done with either ABA or SNP alone (Fig. 1). The synergism observed between ABA and NO may reflect two weak signals in the same pathway that augment each other. Thus, small and rapid changes in both ABA and NO concentrations can determine variations in percentages of stomatal closure and probably explain the spatial and temporal heterogeneity in stomatal behavior, as has been already described (Mott and Buckley, 2000).

Although this assay presented an evidence of a putative ABA-NO interaction, to test if endogenous NO also takes part of the ABA signaling pathway, we tested the effect of the specific NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO) on the ABA-induced stomatal closure. Figure 2A shows that in the presence of c-PTIO, the percentage of open stomata remained constant through all the tested ABA concentrations, showing that the guard cells were not responding to ABA treatment. The difference in the pore width between ABA treatment in the presence or absence of c-PTIO can be clearly observed under an optical microscope (Fig. 2B). In addition, when 200 μM SNP was added after the ABA + c-PTIO treatment, the stomatal closure was induced again, showing that the c-PTIO-mediated inhibition of ABA-induced stomatal closure is reversible (Fig. 2A, inset). All together, these data suggest that NO might be acting downstream of the ABA-induced signaling cascade.

Given the cross talk between ABA and NO, we wanted to know the variations of endogenous NO during the ABA-induced stomatal closure. With that aim, epidermal strips were loaded with the permeable NO sensitive fluorophore 4,5-diaminofluorescein diacetate (DAF-2 DA), which allows the

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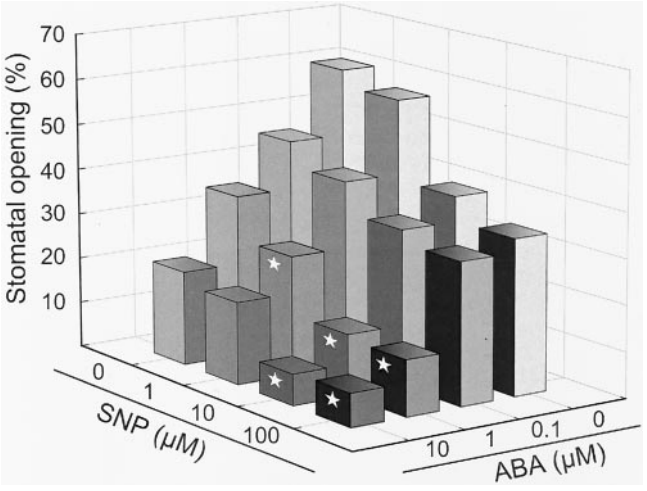

Figure 1. NO involvement in ABA-induced stomatal closure. *V. fava* epidermal strips were pre-incubated for 1 h in opening buffer (10 mM MES, pH 6.1, and 10 mM KCl) and then treated for 1 h with ABA (0, 0.1, 1, and 10 μM) in the presence of different concentrations of SNP (0, 1, 10, and 100 μM). Stomatal opening values (counted under optical microscope 400×) are expressed as mean ± SE (30 stomata populations, taken from at least three independent experiments). Stars mean significant difference with $P < 0.05$ (Student's *t* test).

detection of NO presence in both animal and plant cells (Kojima et al., 1998; Foissner et al., 2000). DAF-2 DA produced a slight accumulation of green fluorescence during control treatments (opening buffer, mode of the green pixel intensity [mgpi]: 24.5 ± 1.3; Fig. 3A). In contrast, when DAF-2 DA was added after the ABA treatment, there was a clear increase in the green fluorescence (mgpi: 46.5 ± 3.1), not only near the stomatal pore, but also spread out all along the guard cells, evidencing the cell limits (Fig. 3B, inset). Because DAF-2 DA fluorescence of ABA + c-PTIO was as low as in the control treatment (mgpi: 28 ± 4.3; Fig. 3C), the increase of DAF-2 DA fluorescence in ABA treatment was due to an endogenous NO accumulation (Fig. 3B). The treatment of epidermal strips with 150 μM SNP (which releases 1 μM NO) produced a green fluorescence level (mgpi: 51.5 ± 3.9) similar to that obtained in the ABA treatment (Fig. 3D). None of the four treatments showed significant increases of green fluorescence levels when loaded with the negative probe 4-AF DA, which lacks one of the amino groups that constitutes the NO specificity domain of the DAF-2 DA molecule (Fig. 3, E–F). This last result confirms that the increase of the green fluorescence corresponds to an accumulation of endogenous NO, and not to unspecific reactions of the probe. In preliminary experiments, epidermal strips from drought stressed *V. fava* plants presented higher levels of NO compared with non-stressed plants (data not shown).

Schroeder et al. (2001b) recently proposed a model involving ABA signal pathways to modulate guard cell responses during water deficit. That model summarizes the components and functions of ion channels and chemical messengers that contribute to cytosolic Ca^{2+} elevation, the loss of guard cell turgor, and finally to stomatal closure. Now, we propose that NO accumulation in *V. fava* guard cells is necessary for the ABA-induced stomatal closure, placing NO as a new component of the ABA signaling transduction pathway during adaptive plant responses to drought. The Arabidopsis mutants *abi1-1* and *abi2-1* are impaired in the ABA-induced stomatal closure, K^+ channel regulation, and ion channel activation. In these mutants, experimental cytoplasmic Ca^{2+} elevation causes anion channel activation and stomatal closure. Thus, it will be interesting to investigate if NO could be able to bypass the effects of *abi1-1* and *abi2-1* mutations (Schroeder et al., 2001a). It is clear that further investigations are needed to find the potential node for the cross talk between ABA and NO interaction.

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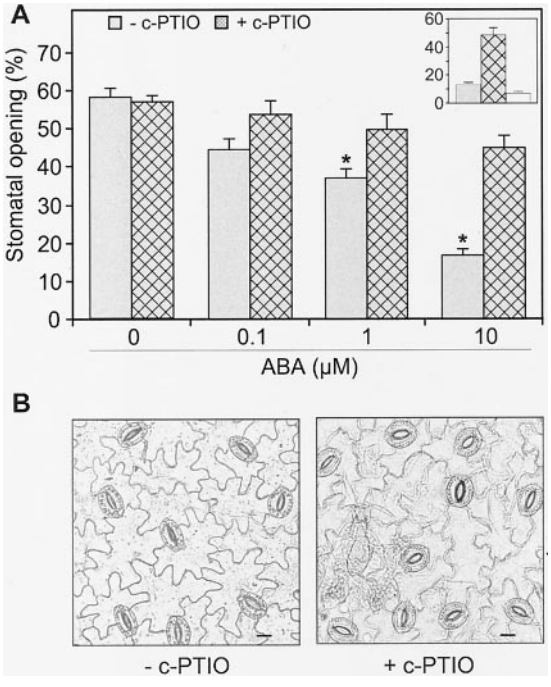

Figure 2. Effect of c-PTIO on ABA-induced stomatal closure: A, Epidermal strips of *V. fava* were pre-incubated for 1 h in opening buffer and then treated with ABA (0, 0.1, 1, or 10 μM) with or without 200 μM c-PTIO. Stomatal opening values (counted under optical microscope 400×) are expressed as mean ± SE (30 stomata populations, taken from at least three independent experiments). Asterisks mean significant differences with $P < 0.05$ (Student's *t* test). Inset, Strips were treated with 10 μM ABA (▩) or 10 μM ABA + 200 μM c-PTIO (☒) or 10 μM ABA + 200 μM c-PTIO for 1 h and then 200 μM SNP was added to the solution (□). B, Bright-field image of a 10 μM ABA treatment in presence or absence of 200 μM c-PTIO. Bars = 10 μm.

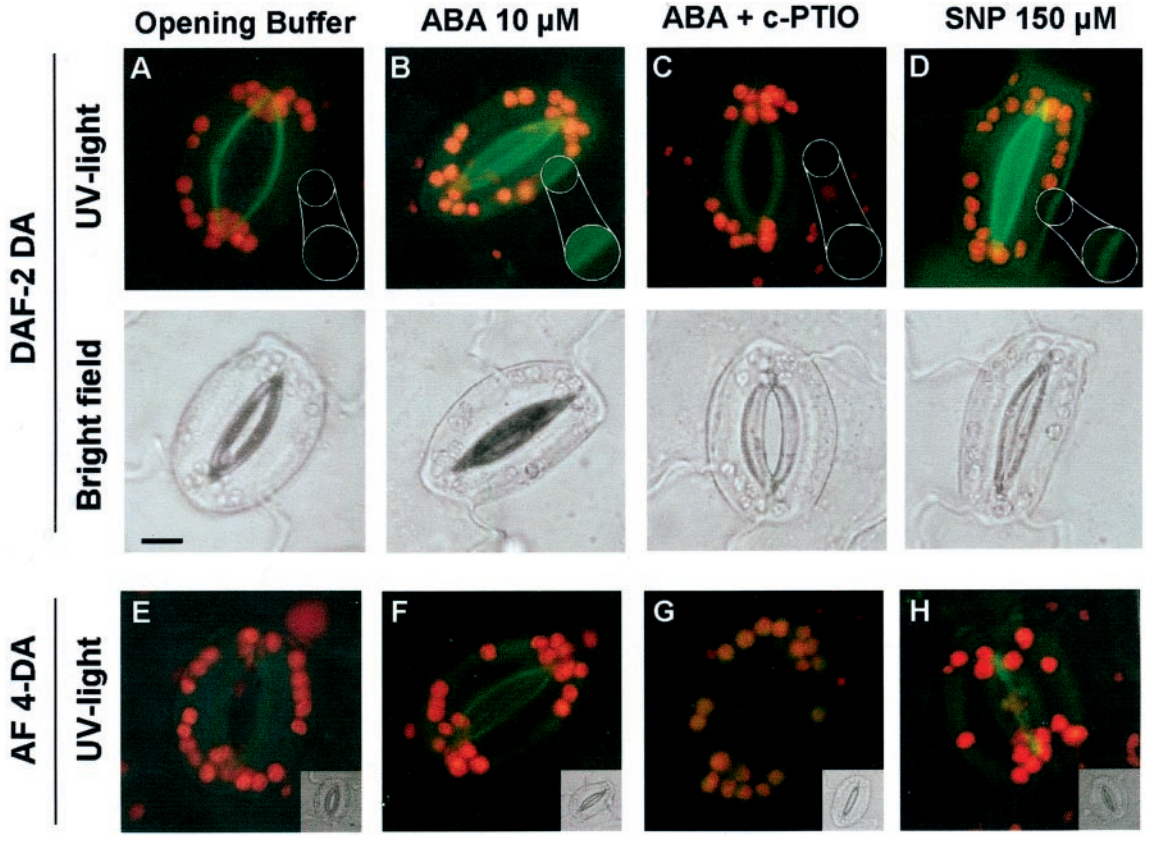

Figure 3. DAF-2 DA fluorescence accumulation in *V. fava* guard cells. Epidermal strips treated with 10 μM ABA or 10 μM ABA plus 200 μM c-PTIO or 150 μM SNP were loaded for 30 min with one of the fluorescent probe, and then thoroughly washed to remove the excess of probe. A through D, Treatments loaded with DAF-2 DA. Insets show magnifications of the guard cell area marked with the small circle. E through F, Treatments loaded with the negative probe 4-amino fluorescein diacetate (4-AF DA). Insets show the bright-field image of the same stomata. Green fluorescence (505–530 nm) corresponds to DAF-2 DA or 4-AF DA; red fluorescence corresponds to chlorophyll autofluorescence. Scale bars = 5 μm.

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